

Directed Evolution of *Vibrio fischeri* LuxR for Improved Response to Butanoyl-Homoserine Lactone[†]

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LuxR is the 3-oxohexanoyl-homoserine lactone (3OC6HSL)-dependent transcriptional activator of the prototypical acyl-homoserine lactone (AHL) quorum-sensing system of *Vibrio fischeri*. Wild-type LuxR exhibits no response to butanoyl-HSL (C4HSL) in quantitative bioassays at concentrations of up to 1 μ M; a previously described LuxR variant (LuxR-G2E) exhibits a broadened response to diverse AHLs, including pentanoyl-HSL (C5HSL), but not to C4HSL. Here, two rounds of directed evolution of LuxR-G2E generated variants of LuxR that responded to C4HSL at concentrations as low as 10 nM. One variant, LuxR-G4E, had only one change, I45F, relative to the parent LuxR-G2E, which itself differs from the wild type at three residues. Dissection of the four mutations within LuxR-G4E demonstrated that at least three of these changes were simultaneously required to achieve any measurable C4HSL response. The four changes improved both sensitivity and specificity towards C4HSL relative to any of the other 14 possible combinations of those residues. These data confirm that LuxR is evolutionarily pliable and suggest that LuxR is not intrinsically asymmetric in its response to quorum-sensing signals with different acyl-side-chain lengths.

Bacterial cell-cell communication employs specific protein receptors, soluble pheromones, and is frequently referred to as quorum sensing (QS) (35). Numerous proteobacteria employ acyl-homoserine lactones (AHLs) as dedicated pheromones to regulate genes in a population density-dependent manner (16, 17, 31). In the *lux* system of *Vibrio fischeri*, 3-oxohexanoyl-homoserine lactone (3OC6HSL) is synthesized by LuxI and is sensed by LuxR, a DNA-binding transcriptional activator (11). LuxR activates transcription of the *lux* genes when concentrations of 3OC6HSL reach ca. 10 nM within the cell (5, 11, 22). Through heterologous genetic and whole-cell biochemical techniques, amino acid residues of LuxR that are required for AHL binding, DNA binding, dimerization, and recruitment of RNA polymerase have been identified (13, 15, 16, 30, 33). Detailed structural information for LuxR is not yet available, as this protein has only recently been purified in its full-length form (34).

There has been considerable interest in understanding how receptor proteins distinguish among a myriad of potential ligands (3, 8–10, 24, 28, 29). LuxR homologs generally are reasonably specific and sensitive to their specific cognate AHLs (36). Collins et al. employed directed evolution to yield LuxR variants that exhibited an improved response to AHLs with longer acyl side chains (8). One variant, LuxR-G2E, was equally as sensitive to octanoyl-HSL (C8HSL) as wild-type LuxR was to 3OC6HSL. Recently, a positive-negative, dual-

selection system was employed to generate a C8HSL-responsive variant of LuxR that was no longer sensitive to 3OC6HSL (9). Curiously, wild-type LuxR exhibits a weak but measurable response to C8HSL, C10HSL, and even C12HSL but none to C5HSL or C4HSL. The broadened-specificity variant LuxR-G2E exhibited a strong response to C5HSL (8) but none to C4HSL. This suggests that the native specificity of LuxR for AHLs is broad yet asymmetric with respect to acyl-side-chain length (12, 26). C4HSL serves as the cognate signal for both RhlR and AsaR, homologs of LuxR operative in the bacteria *Pseudomonas aeruginosa* and *Aeromonas* species (25, 32). RhlR and AsaR are only distantly related to LuxR, and the signal binding domains of these proteins share less than 23% identity with LuxR over a span of 160 residues. Herein, we endeavored to examine whether the previously observed plasticity of LuxR can be extended to C4HSL or whether, conversely, the observed asymmetric response of LuxR to a full range of signals with different acyl chain lengths is insurmountable.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The strains and plasmids used in this study are listed in Table 1. All strains were grown on rich medium (LB) at 37°C, unless otherwise noted. Liquid medium was solidified with 1.5% agar (Becton Dickinson and Co., Franklin Lakes, NJ). Antibiotics were used at the following concentrations, where appropriate: kanamycin, 20 μ g per ml; and chloramphenicol, 100 μ g per ml. Bioassay medium was used as previously described (27). Competent *Escherichia coli* cells were prepared with the Z-Competent kit according to the manufacturer's instructions (Zymo Research, Orange, CA). AHL stock solutions were prepared by dissolving the appropriate amount of each in ethyl acetate acidified with 0.01% (vol/vol) glacial acetic acid. AHLs and AHL analogues used in this study were either synthesized (see below) or acquired from the following sources: 3OC6HSL and acetyl-homocysteine-thiolactone (AHCTL) were from Sigma Aldrich, St. Louis, MO; and DL-C8HSL, DL-C10HSL, DL-C12HSL, and DL-C14HSL were from Fluka, St. Louis, MO. All AHLs used in this study were enantiopure L isomers, unless otherwise indicated.

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TABLE 1. Strain and plasmids used in this study

Strain or plasmid	Relevant characteristics and/or nucleotide changes ^a	Source or reference
Strain		
<i>E. coli</i> DH5α	F ⁻ λ ⁻ <i>recA1</i> Δ(<i>lacZYA-argF</i>) U169 <i>hsdR17</i> (r _k ⁻ m _k ⁺) <i>thi-1</i> <i>gyrA96</i> <i>supE44</i> <i>endA1</i> <i>relA1</i> φ80d <i>lacZ</i> Δ <i>M15</i> <i>phoA</i> <i>deoR</i>	8
Plasmids		
pPROLar.A122	Km ^r ; expression plasmid containing P _{<i>lac/ara-1</i>} ; P15A <i>ori</i>	BD Biosciences, Clontech
pLuxR	Km ^r ; wild-type LuxR expression plasmid; P15A <i>ori</i>	8
pLuxGFPuv	Cm ^r ; GFPuv under control of P _{<i>lux</i>} ; ColE1 <i>ori</i>	8
pLuxR-G2E	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, T162A*, T346G, G405A	8
pLuxR-G3A	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A102G*, T162A*, T346G, G405A, A433G, T436A, T668A	This study
pLuxR-G3B	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, T162A*, A257G, T346G, T360C*, G405A, A520G	This study
pLuxR-G3C	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A117T, T162A*, A343G, T346G, G405A, T651C*	This study
pLuxR-G3D	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, T162A*, T346G, T360A*, G405A, A470G, A503G	This study
pLuxR-G3E	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, T162A*, A177T*, A343G, T346G, G405A, A512G	This study
pLuxR-G3F	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, T162A*, A294G*, T346G, G405A, T440A, T490A, A679G	This study
pLuxR-G3G	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T162A*, T346G, A358G, G405A, A431G, A458G, T497C	This study
pLuxR-G3H	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, T111A, A117T, A156G, T162A*, T346G, G405A, T459C*, A472G, T606C*, A707G, T714C*	This study
pLuxR-G3I	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A146G, T162A*, A273G*, T346G, G405A, A470G, A545G, A684G*, A696T	This study
pLuxR-G3J	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T162A*, T240A*, T346G, G405A, T454C*, A495T*, A526G, A628T, A696T	This study
pLuxR-G4A	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T162A*, T240A*, T346G, A358G, G405A, A545G	This study
pLuxR-G4B	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T162A*, T346G, G405A, A495T*, A520G	This study
pLuxR-G4C	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T162A*, T346G, G405A, A431G, A458G, A696T	This study
pLuxR-G4D	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T162A*, T346G, G405A, A503G	This study
pLuxR-G4E	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T346G, G405A, A495T*	This study
pLuxR-G4F	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T162A*, T346G, G405A, T440A, T651C*	This study
pLuxR-G4G	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A133T, T162A*, T346G, G405A, A472G	This study
pLuxR-G4H	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, T111A, A117T, T162A*, A257G, A343G, T346G, T360A*, G405A, A520G, A707G, T714C*	This study
pLuxR-G4I	Km ^r ; <i>luxR</i> -directed evolution mutant; A97G, A102G*, T162A*, A133T, A177T*, A343G, T346G, G405A, A520G, A526G, A679G	This study
pLuxR T33A	Km ^r ; <i>luxR</i> site-directed mutant; A97G	8
pLuxR I45F	Km ^r ; <i>luxR</i> site-directed mutant; A133T	This study
pLuxR S116A	Km ^r ; <i>luxR</i> site-directed mutant; T346G	8
pLuxR M135I	Km ^r ; <i>luxR</i> site-directed mutant; G405A	8
pLuxR T33A I45F	Km ^r ; <i>luxR</i> site-directed mutant; A97G, A133T	This study
pLuxR T33A S116A	Km ^r ; <i>luxR</i> site-directed mutant; A97G, T346G	This study
pLuxR T33A M135I	Km ^r ; <i>luxR</i> site-directed mutant; A97G, G405A	This study
pLuxR I45F S116A	Km ^r ; <i>luxR</i> site-directed mutant; A133T, T346G	This study
pLuxR I45F M135I	Km ^r ; <i>luxR</i> site-directed mutant; A133T, G405A	This study
pLuxR S116A M135I	Km ^r ; <i>luxR</i> site-directed mutant; T346G, G405A	This study
pLuxR T33A I45F S116A	Km ^r ; <i>luxR</i> site-directed mutant; A97G, A133T, T346G	This study
pLuxR T33A I45F M135I	Km ^r ; <i>luxR</i> site-directed mutant; A97G, A133T, G405A	This study
pLuxR T33A S116A M135I	Km ^r ; <i>luxR</i> site-directed mutant; A97G, T346G, G405A	This study
pLuxR I45F S116A M135I	Km ^r ; <i>luxR</i> site-directed mutant; A133T, T346G, G405A	This study

^a Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; *ori*, origin of replication. Nucleotides are given according to the one-letter codes and are listed according to the wild-type *luxR* position. An asterisk indicates that the nucleotide change does not result in a change to the deduced amino acid sequence.

For library screening, AHL was added to agar medium cooled to 65°C before being poured into petri plates. All liquid media containing AHLs were prepared immediately prior to use by adding the appropriate amount of acidified ethyl acetate stock directly to the growth medium. Ethyl acetate was always ≤0.2%

(vol/vol) final concentration, and control experiments showed that this concentration of ethyl acetate had no effect on *E. coli* growth (not shown).

Enantiopure AHL synthesis. A 100-ml round-bottom flask was charged with 50 ml dichloromethane and 2.5 g (13.7 mmol) α-amino-γ-butyrolactone-hydro-

bromide; the suspension was cooled to 0°C. Subsequently, 2.77 g (27.4 mmol) triethylamine was added dropwise. Afterwards, a small spatula tip (approximately 50 mg) of 4-dimethylaminopyridine was added, followed by 1.84 g (13.7 mmol) hexanoyl-chloride. The mixture was allowed to reach room temperature and was stirred overnight. To the mixture was added 10 ml of 0.5 M HCl. The phases were separated, and the organic phase was washed with 6 ml of water and 10 ml of a 5% sodium bicarbonate solution. After drying the organic phase with sodium sulfate, the solvent was removed under vacuum. The yield was 50% of the theoretical 2.63 g. *N*-Hexanoyl-L-HSL had a purity of >95% as judged by 500-MHz ¹H-nuclear magnetic resonance (NMR) data. The NMR data obtained were in accordance with previous reports (6, 23). Other *n*-alkanoyl derivatives were synthesized in a similar fashion.

Directed evolution library generation, screening, and mutant verification. As described previously (8), *luxR* variants were cloned into the expression vector pPROLar.A122 and screened in combination with the signal response screening plasmid *pluxGFPuv*. Use of pPROLar.A122 places cloned *LuxR* alleles under the control of the hybrid *P_{lacIara-1}* promoter. Plasmid *pluxGFPuv* encodes a variant of green fluorescent protein (GFP) under the control of the *P_{luxI}* promoter.

Error-prone PCRs were performed using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) and 50 mM MnCl₂ to increase the mutation rate as described previously (7). The primers 5'-*luxR* and 3'-*luxR* were used to amplify the *luxR-G2E* gene using pLUXR-G2E as the template (8). The library was constructed by ligating KpnI- and BamHI-digested pPROLar.A122 with the products of error-prone PCR using T4 DNA ligase (Invitrogen, Carlsbad, CA). Vent DNA polymerase (New England Biolabs, Beverly, MA) was used to amplify wild-type *luxR*, which was digested and ligated into pPROLar.A122 for use as a control. The ligation mixtures were transformed into competent DH5α cells harboring *pluxGFPuv*. Libraries were screened for GFPuv fluorescence under 365-nm UV light on LB agar plates containing 1 μM C4HSL. Screening plates were buffered at pH 6.5 using either phosphate or 3-(*N*-morpholino)-propanesulfonic acid. The plates were incubated at 37°C for 18 h prior to screening.

DNA shuffling was performed as described previously (21). The primers 5'-*luxR* and 3'-*luxR* were used to amplify the mutant *luxR* genes using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). After purification and quantification, equal amounts of parent amplification products were mixed and subjected to a DNase I digestion. The 100-μl digest contained ca. 4 μg of the parental mix, 10 μl of 0.5 M Tris-HCl (pH 7.4), 5 μl of 0.2 M manganese chloride, and 0.14 U of DNase I. After 15 to 30 s of digestion at 15°C, each reaction was stopped by adding 5 μl of 0.5 M EDTA and by placing the mixture immediately on ice and was subjected to electrophoresis through a 1.5% agarose gel. Fragments between 50 and 150 bp were excised and used in further steps. Fragments were randomly reassembled in a 50-μl reaction. Full-length *luxR* genes were synthesized by diluting the reassembly reaction 50- to 500-fold and amplified using *Pfu* Turbo DNA polymerase and the primers 5'-*luxR* and 3'-*luxR*. Ligations and transformations were performed similarly to the first generation. The resultant library was screened for GFPuv fluorescence under 365-nm UV light on LB agar plates containing 100 nM C4HSL. Screening plates were buffered at pH 6.5 using either phosphate or 3-(*N*-morpholino)-propanesulfonic acid. All colonies that fluoresced after 18 h were picked, purified, and inoculated into 1 ml LB containing the appropriate antibiotics before further characterization.

Mutants identified during both screens were recloned into fresh background plasmids and strains to eliminate secondary site effects. For each mutant, the *luxR* allele was amplified using *Pfu* Turbo polymerase and treated with DpnI. The PCR products were digested and ligated into pPROLar.A122 (as described above) and transformed into competent DH5α cells containing *pluxGFPuv*. The promoter and *luxR* gene from all mutants of interest were sequenced using the upstream primer 5'-LarSeq2 (5'-CCTGAGCAATCACCTATGAACTGTC-3') and internal *luxR* primer LuxRSeq(int) (5'-CGAAAACATCAGGTCTTATCACTGGG-3').

Site-directed mutagenesis. Single mutants were constructed previously (8), and double and triple mutants were constructed by overlap extension PCR, as previously described (18, 19). Primer sequences are available upon request. Mutations were verified by DNA sequencing with at least twofold coverage.

Quantitative LuxR-mediated GFPuv expression. The bioassay used to quantify the activity of each *LuxR* mutant protein has been previously described (8, 27). Cells were first grown from single colonies or glycerol stocks in LB overnight and then diluted 200-fold into 100 ml of fresh LB medium containing 5 mM potassium phosphate buffer (pH 6.5) and the appropriate antibiotics. Such cultures were incubated with shaking at 37°C until they reached an optical density at 600 nm (OD₆₀₀) of 0.5 and then harvested by centrifugation. Cell pellets were washed and resuspended to an OD₆₀₀ of 0.6 in bioassay medium (0.05% [wt/vol] tryptone, 0.03% [vol/vol] glycerol, 100 mM sodium chloride, 50 mM magnesium

sulfate, and 5 mM potassium phosphate buffer [pH 6.5]) containing antibiotics. The suspension was subsequently transferred into 48-well plates (VWR International, catalog no. 82004-674) containing 0.5 ml bioassay medium with acyl-HSL, to a total volume of 2.5 ml per well. Thereafter, the 48-well plates were shaken at 37°C for 4 h. From each well, 200 μl was transferred to wells of a white 96-well microplate with a clear bottom. GFPuv fluorescence (395-nm excitation, 509-nm emission, 495-nm cutoff) was measured using a fluorescence microtiter plate reader (Molecular Devices, SpectraMAX Gemini XS); cell densities were measured using a microtiter plate reader at 600 nm. Fluorescence by cell suspensions was normalized to OD. The fluorescence output of pPROLar.A122 with *pluxGFPuv* was used to determine the background fluorescence without *LuxR*, and this background fluorescence value was subtracted from all fluorescence measurements obtained with wild-type *pLUXR* and all mutants to determine fluorescence due only to *LuxR*-dependent gene activation. For a given variant, the same batch of cells was used for comparing responses to (i) different concentrations of a given acyl-HSL or (ii) different acyl-HSLs.

AHL sensitivity and specificity calculations. Data from AHL dose-response curves were plotted using the OriginPro software package, version 7.5 SR4 (OriginLab Corporation, Northampton, MA). The half-maximal gene activation values, e.g., [AHL]₅₀, were calculated by fitting the data to the best-fit model using regression analysis. Data were fit using either a hyperbolic or a linear equation, and these equations were used to calculate the half-maximal gene activation value for each AHL. Half-maximal gene activation values were used as a measure of sensitivity toward a particular AHL. Relative specificity, *S*, was determined by calculating the ratio of half-maximal activation values for each AHL compared to the cognate AHL, L-3OC6HSL, as described previously (8). For example, the relative specificity of L-C4HSL was calculated as follows: $S_{C4HSL} = [L-C4HSL]_{50} / [L-3OC6HSL]_{50}$. Mutants that have an *S* value of 1 are as sensitive to the test AHL as to L-3OC6HSL. Mutants that are more sensitive to L-3OC6HSL than to the test AHL have an *S* value of greater than 1. An *S* value of less than 1 means a particular mutant is more sensitive to the test AHL than to L-3OC6HSL. *S* values were used as a measure of specificity for all generated mutants.

Western immunoblots and quantitative densitometry. Each *LuxR* mutant protein was tagged with a *c-myc* tag (GGAEQKLISEEDL) as described previously (8). Cells for Western immunoblots were grown exactly like those for quantitative *LuxR*-mediated GFPuv expression and incubated with or without AHL, as in quantitative bioassays. Cells were harvested by centrifugation, washed once, and resuspended in 100 μl ultrapure water (Fluka, St. Louis, MO) to which 50 mg/ml CellLytic Express (Sigma-Aldrich, St. Louis, MO) was added. Suspensions were incubated at 37°C for 10-min intervals with intermittent vortexing until the suspension appeared clear and cells were lysed. Residual cell material was removed by centrifugation in a microcentrifuge at maximum speed (16,000 × *g*), and the cleared lysate was transferred to a new tube. The total protein concentration was determined with the Bio-Rad protein assay (Hercules, CA). Equal amounts of each sample were loaded onto a NuPAGE 10% Bis-Tris gel (Invitrogen Life Technologies, Carlsbad, CA) and separated by electrophoresis. Protein was transferred to polyvinylidene difluoride membranes (Invitrogen Life Technologies, Carlsbad, CA), and *c-myc*-tagged *LuxR* proteins were detected using anti-*c-myc* primary antibody (Roche Diagnostics, Indianapolis, IN) and the WesternBreeze chemiluminescent immunodetection kit (Invitrogen Life Technologies, Carlsbad, CA). Blots were exposed to Kodak BioMax film (Rochester, NY). The developed film was scanned using a Hewlett Packard Scanjet 4570c scanner and the included "transparent materials adapter." Images were analyzed using ImageJ (<http://rsb.info.nih.gov/ij/>) with the "subtract background" feature applied.

RESULTS

Directed evolution of LuxR for response to C4HSL. *LuxR-G2E*, a *LuxR* allele that contains three amino acid changes compared to the wild type (T33A, S116A, and M135I) was chosen as the parent allele for this study because it exhibited a response to a synthetic short-chain AHL (C5HSL), whereas *LuxR* did not (8). By screening a random PCR mutagenesis library of *LuxR-G2E* for response to C4HSL, 10 "third-generation" mutants with an average of 7.6 additional mutations per allele (6.2 nonsynonymous mutations) were identified (Fig. 1A and Table 1). DNA shuffling of these third-generation mutants yielded 9 unique "fourth-generation" mutants with an average

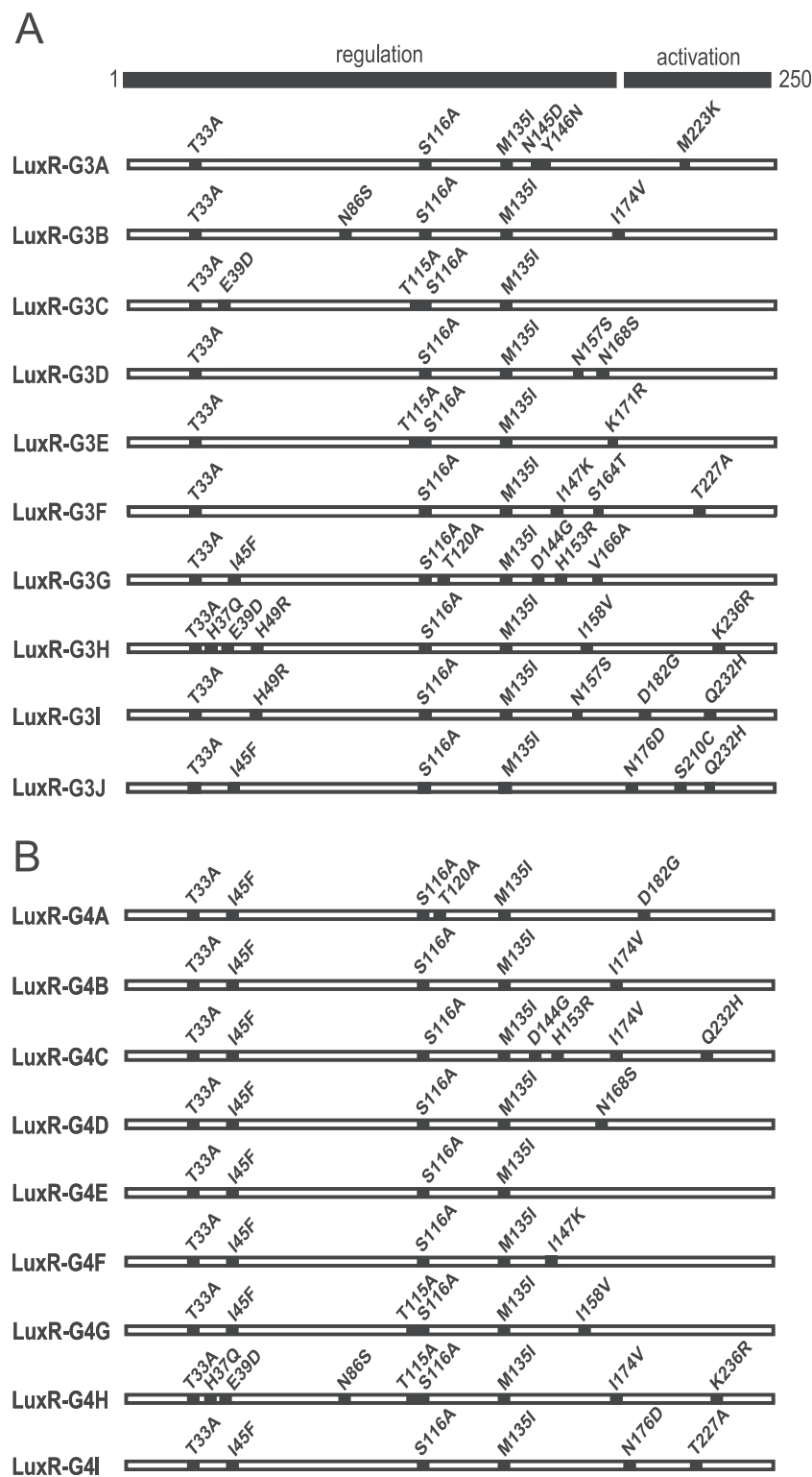


FIG. 1. Deduced amino acid changes in third-generation (A) and fourth-generation (B) LuxR variants responsive to butanoyl-HSL. Amino acids are labeled according to the one-letter codes. Positions are based on a LuxR polypeptide of 250 amino acids in length for ease of reference to the previous literature. However, in a recent study, purified LuxR was shown to be a 252-amino-acid polypeptide (34).

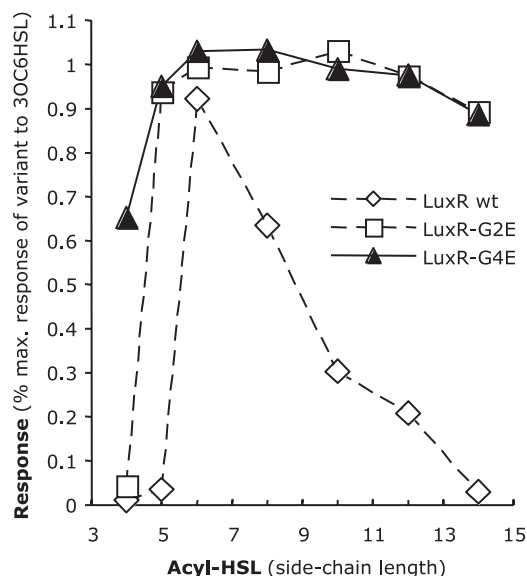


FIG. 2. Relaxed specificity of LuxR-G4E. LuxR-G4E has the most promiscuous response to AHLs, compared to the wild type (wt) and the LuxR-G2E parent. LuxR-G4E has only one additional amino acid change, I45F, compared to the parent, LuxR-G2E. This change permits the response to C4HSL. (The curve has shifted left.) Acetyl-homocysteine thiolactone (C2HCTL), a two-carbon AHL analogue, was not detected by any of the LuxR variants tested.

of 6.8 additional mutations per allele (6.0 nonsynonymous mutations) with respect to wild-type LuxR (Fig. 1B). Neither wild-type LuxR nor LuxR-G2E responded to C4HSL under the conditions used in this study. However, all 19 third- and fourth-generation variants responded to C4HSL (see Fig. S1 and S2 in the supplemental material).

Successive generations of LuxR mutants were more sensitive and specific to short-acyl-chain AHLs. The sensitivity of third- and fourth-generation mutants towards C4HSL, C5HSL, and 3OC6HSL was determined by calculating the half-maximal gene activation value for each mutant and for each AHL (i.e., from dose-response curves [see Materials and Methods and Fig. S1 and S2 in the supplemental material]). Wild-type LuxR required 24 nM 3OC6HSL to achieve half-maximal gene activation but exhibited no observed response to C4HSL or C5HSL. LuxR-G2E required 26 nM 3OC6HSL and 110 nM C5HSL to achieve half-maximal gene activation, with no response to C4HSL observed. Third-generation LuxR mutants required an average of 9 nM 3OC6HSL, 22 nM C5HSL, and 2,300 nM C4HSL for half-maximal gene activation. Fourth-generation mutants required an average of ≤ 1 nM 3OC6HSL, 2 nM C5HSL, and 150 nM C4HSL for half-maximal gene activation (see Tables S1 and S2 in the supplemental material). The improved responses of the LuxR variants towards C4HSL involved, but were not solely a function of, their becoming more sensitive to all AHLs (i.e., in a general manner). A component of the improved response also involved changes in the specificity (*S*) of the variants (i.e., towards C4HSL). For example, by comparing the ratio of the half-maximal responses towards C4HSL versus C5HSL for each variant, an improvement in *S* was observed.

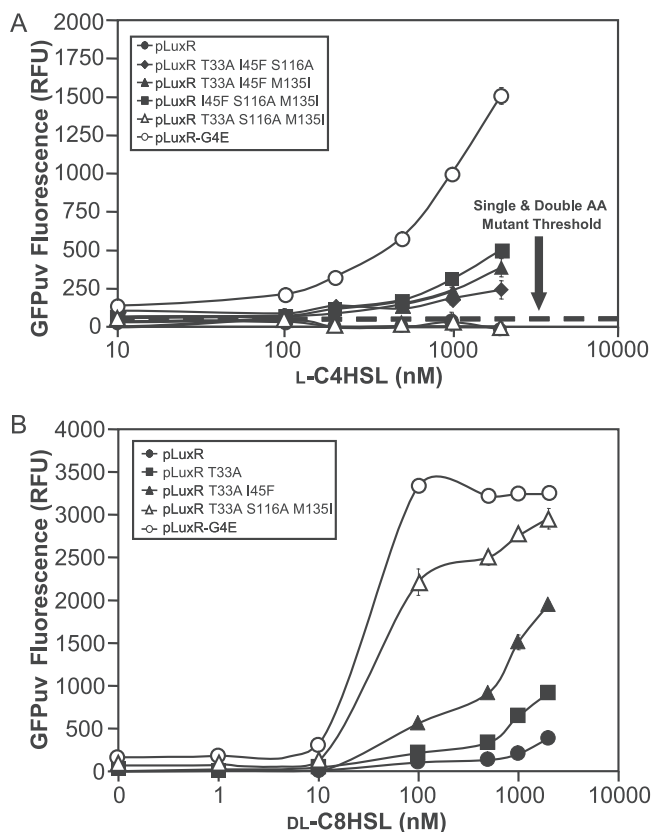


FIG. 3. Dissection of LuxR-G4E mutations. A combination of at least three mutations was required for C4HSL detection, whereas individual mutations increase the response to DL-C8HSL. (A) Mutant response to C4HSL. The heavy dashed line indicates the maximum response observed by any single- or double-amino-acid (AA) mutant, as these are omitted from the graph for clarity. (B) Mutant response to DL-C8HSL. For clarity, only representative mutants are shown. Error bars represent the standard deviation of three technical replicates, and graphs are representative of at least two separate experiments. Please note scale differences. RFU, relative fluorescence units.

LuxR-G4E responded strongly to C4HSL and had only one additional mutation compared to LuxR-G2E. The fourth-generation variant LuxR-G4E had four deduced amino acid substitutions compared to the wild type (T33A, I45F, S116A, and M135I). With the exception of I45F, these mutations were already present in LuxR-G2E, the second-generation variant used as the parent in these studies (Fig. 1B). LuxR-G4E retained broad specificity to AHLs with side chains of 5 to 14 carbons in length, a trait characteristic of its LuxR-G2E parent, which, however, does not respond to C4HSL (Fig. 2).

Dissection of LuxR-G4E revealed that combinations of at least three mutations were required to achieve C4HSL detection. Using site-directed mutagenesis of wild-type LuxR, all possible 15 permutations of the four mutations within LuxR-G4E were constructed. The response of each variant to AHLs was analyzed via quantitative bioassays. Half-maximal gene activation values were determined from dose-response curves for each variant (Fig. 3 and Table 2). Dissection of LuxR-G4E revealed that none of the single amino acid changes, including I45F, or combinations of any two changes permitted detection of C4HSL (Fig. 3A). Three of the four possible triple mutants

TABLE 2. AHL responses of wild-type LuxR and dissected variants of LuxR-G4E

LuxR allele and type of mutant	Amino acid change(s)	Sensitivity (nM) ^a			Specificity ^b	
		[3OC6HSL] ₅₀	[dl-C8HSL] ₅₀	[C4HSL] ₅₀	S _{C8HSL}	S _{C4HSL}
LuxR (wild type)		24	7,900	NR	330	—
LuxR mutant						
Single	T33→A	16	1,600	NR	100	—
	I45→F	9	2,900	NR	320	—
	S116→A	15	1,700	NR	110	—
	M135→I	120	5,300	NR	44	—
Double	T33→A, I45→F	7	980	NR	140	—
	T33→A, S116→A	7	1,200	NR	171	—
	T33→A, M135→I	85	820	NR	10	—
	I45→F, S116→A	5	860	NR	170	—
	I45→F, M135→I	34	630	NR	19	—
	S116→A, M135→I	110	1,200	NR	11	—
Triple	T33→A, I45→F, S116→A	3	100	28,000	29	8,200
	T33→A, I45→F, M135→I	11	54	14,000	5	1,300
	T33→A, S116→A, M135→I	30	68	NR	2	—
	I45→F, S116→A, M135→I	9	41	10,000	5	1,100
LuxR-G4E	T33→A, I45→F, S116→A, M135→I	2	29	180	12	75

^a [3OC6HSL]₅₀, [DL-C8HSL]₅₀, and [C4HSL]₅₀ are the nM concentrations required of each AHL listed to achieve half-maximal gene activation, as measured by fluorescence intensity of the GFPuv reporter. NR, no response after incubation with 10 μM C4HSL. Values were calculated as described in Materials and Methods and represent at least two replicates. Standard deviations were within ±18%.

^b The specificity constants, S_{C8HSL} and S_{C4HSL}, are a measure of relative specificity for the given AHL as compared to the cognate signal, 3OC6HSL (see Materials and Methods for further explanation). —, variant has no response, and thus no specificity, for the given AHL.

exhibited a weak response to C4HSL (Fig. 3A and Table 2) but were 2 orders of magnitude less sensitive to C4HSL relative to LuxR-G4E (Table 2).

Western immunoblots and quantitative densitometry. All site-directed and fourth-generation mutants (including LuxR-G4E) were subjected to analysis by whole-cell-lysate Western immunoblots and quantitative densitometry. Across the board, increases in sensitivity of each mutant to 1-3OC6HSL correlated well with higher steady-state protein levels. However, as has been discussed previously, although increases in protein concentration can result in generalized increases in the response of LuxR to all AHLs, such increases cannot account for changes in specificity. The mutant dissection results (Table 2) reveal that C4HSL specificity has been significantly altered (improved) in the all of the variants exhibiting a response to this AHL, with the greatest improvement observed with the 4 amino acid changes of LuxR-G4E.

DISCUSSION

Wild-type LuxR exhibits a greater response to AHLs with acyl side chains up to six carbons longer than that to its cognate signal (3OC6HSL) than it does to those even one carbon shorter (Fig. 2). However, the results here demonstrate that there is no intrinsic or insurmountable barrier to broadening the response of LuxR to short-chain QS signals such as C4HSL.

The mutation I45F was recovered with great frequency among fourth-generation variants, and two of the third-generation mutants also contained this change (Fig. 1). The I45F change was shown to be essential for C4HSL detection by LuxR-G4E, but alone it was not sufficient to permit C4HSL detection when re-

constructed into a wild-type background (Fig. 3). However, the single mutation alone did yield improved responses to C8HSL and 3OC6HSL (Table 2). This likely explains why this mutation has been observed previously, when mutations that improved LuxR response to C8HSL were identified (8).

In the specific variant studied here, three or more combinations of mutations at different locations within the *luxR* primary sequence were required for C4HSL detection. We note that several of the third- and fourth-generation variants in this study exhibited a response to C4HSL but did not contain the I45F change (e.g., LuxR-G4H) (Fig. 1). This indicates that at least one other solution permitting C4HSL detection and likely many more exist. Moreover, it is not necessarily the case that multiple changes are required to gain a strong C4HSL response. Since this study used as starting material a LuxR variant that already involved changes at three residues and since the search for C4HSL-responding variants was by no means exhaustive, the possibility remains that there could yet be many alternative solutions, even several involving only single changes. Nevertheless, it seems notable that as few as four residue changes can result in a strong response and markedly improved specificity of LuxR towards C4HSL. This molecule serves as the cognate signal for both RhlR and AsaR (25, 32), proteins having autoinducer binding domains that each differ from that of LuxR by over 120 residue changes over a span of 160 residues.

Directed evolution has been used extensively to modify enzyme substrate specificity, chiefly for biotechnological applications (4, 14, 37). Directed evolution has become a tool to study the evolution of new protein function in the laboratory and has led to support of the hypothesis that protein evolution occurs

through promiscuous intermediates (1, 2, 20). Using the approaches of directed evolution applied to LuxR as demonstrated here and previously (9), it is plausible to consider that a wide diversity of different QS response regulator variants can be evolved rapidly from LuxR, i.e., to perceive and respond to a multitude of both natural and perhaps even nonnatural AHL structures.

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